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Award Number: W81XWH-10-1-0914

TITLE: Shock Wave-Stimulated Periosteum for Cartilage Repair

PRINCIPAL INVESTIGATOR: Myron Spector, Ph.D.

CONTRACTING ORGANIZATION: Boston VA Research Institute
Boston, MA 02130

REPORT DATE: March 2015

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
March 2015	Final Report	30Sep2010 – 1Dec2014

4. TITLE AND SUBTITLE
Shock Wave-Stimulated Periosteum for Cartilage Repair

5a. CONTRACT NUMBER
W81XWH-10-1-0914

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Myron Spector, Ph.D.; Hu-Ping Hsu, M.D.

email: mspector@partners.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Boston VA Research Institute
150 S. Huntington Ave.
Boston, MA 02130

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The primary objective of this project is to determine if extracorporeal shock wave (ESW)-stimulated periosteum improves cartilage repair when it is used as an autograft to fill a defect in the articular surface of goats. A miniature fiber optic pressure sensor will be inserted into the tibial periosteum of 6 animals to measure the actual shock waveform in the tissue for two ESW doses (energy densities). In 12 goats, tibial periosteum stimulated by one of the 2 doses of ESWs (n=6) will be harvested, 4 days post-treatment, as an autograft for implantation into one 1 cm2 defect surgically produced in the trochlear groove of the knee joint of the same goat. Non-ESW-treated periosteum will serve as the control group (n=6). All animals will be sacrificed after 16 weeks, and the reparative tissue will be quantified histomorphometrically by determining the areal percentage of selected tissues in the original cartilage defect area.

15. SUBJECT TERMS
Shock waves, cartilage, goat

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	**U**	**U**	**UU**	**18**	**19b. TELEPHONE NUMBER** *(include area code)*

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I. INTRODUCTION

The primary objective of this project was to determine if extracorporeal shock wave (ESW)-stimulated periosteum improves cartilage repair when it is used as an autograft to fill a defect in the articular surface of goats. This protocol deals with the therapeutic use of ESWs, which are pressure waves of very short duration (a few microseconds). The initial peak compressive wave is followed by a lower amplitude tensile wave. Normally treatments apply up to 3000 shocks in a session. The "dose" of shock waves is measured in energy density. For our studies the energy densities were: 0.15 mJ/mm^2 ("low") and 0.45 mJ/mm^2 ("high"). This range is approved by FDA for other indications than the one we investigated, but it demonstrates that shock waves in this dose range have an acceptable safety profile.

Periosteum, which contains cells with chondrogenic potential, has been investigated as an autograft for cartilage repair procedures. However, this approach is limited because the cambium layer of the periosteum is normally only 2-5 cells thick, and some of these cells are lost during the harvest procedure. We previously demonstrated that ESWs, at doses approved by FDA for treatment of certain disorders, can stimulate up to a 10-fold increase in the thickness of rat[1] and rabbit[2] periosteum after only 4 days.

One of the principal delays in our performing the DOD study relates to the shock wave apparatus that was employed. In our prior rat and rabbit studies, we employed a commercially-available, focused shock wave apparatus on loan from the manufacturer, which employed the detonation of a spark to create the shock wave which was focused by a parabolic reflector. That apparatus was subsequently taken out of service by the manufacturer. According to the literature, similar results should have been expected from: 1) an unfocused (radial) shock wave apparatus; and 2) a focused apparatus employing piezoelectric crystals to create the focused shock wave. During the initial period of this DOD project we conducted pilot experiments, using goats on other studies, with these radial and piezoelectric apparatus to assess their effects in stimulating periosteal proliferation in goats after 4 days. We did not find the expected increase in the cell number and thickness of periosteum, as we found in the rat and rabbit studies using the initial shock wave apparatus. Therefore, we decided to obtain the newer version of the original focused shock wave apparatus. But we also found that the original focused ESW apparatus that had generated positive findings in the rat and rabbit did not have as dramatic effects on goat periosteum. We did find, however, that: 1) *in vitro*, ESWs stimulated mesenchymal stem cells (MSCs); and 2) *in vivo*, ESWs stimulated a regenerative response in goat marrow. These positive results are being used to formulate a new study to investigate the benefits of ESWs in stimulating a cartilage repair procedure, "microfracture," based on ESW stimulation of marrow.

II. BODY

Specific Aim #1

Task 1. Measurement of the ESW pressure waveform in the periosteum of goats

1.a. Measure the pressure waveforms for select settings of the ESW apparatus in a water bath, for specific locations of sensor away from the head of the ESW device.

1.b. Insert a pressure sensor into the periosteum of the right proximal tibia of 6 goats to measure the pressure waveform in the periosteum for select settings of the ESW apparatus.
Instead of performing this task, which would have necessitated the sacrifice of 6 goats, measurements were made in tissues excised from rabbits being sacrificed on other studies.

A. Measurements of Pressure Waves in Bones *Ex Vivo*

The pressure wave forms for the shock wave apparatus (EMS Dolorclast, Switzerland) were recorded and are reported below, with the specific goal of interrogating the waveform that the periosteum is exposed to during treatment. The Dolorclast was used with the 15mm unfocused convex applicator tip, and was operated at 4bar, which is quoted as being an energy flux density of 0.18mJ/mm^2. Three sets of measurements were taken: (1) free field (water bath) (Fig. 1); (2) in tissue immediately post-sacrifice (intramuscularly and at tibial bone corner); and (3) at the bone surface *ex vivo* (Fig. 2). Measurements were taken using the Onda HNS-0500 needle hydrophone (ONH; Onda Corporation, Sunnyvale, CA) and a polyvinylidene diuoride (PVDF) bilaminar shielded membrane hydrophone (UW Membrane, University of Washington). A Gorillapod (Joby, San Francisco, CA) was adapted to attach the needle hydrophone on one leg to allow multidimensional positioning

of the needle hydrophone. Six measurements were taken for each set-up, and measurements are reported as mean ± standard error of the mean.

Dolorclast measurements at bone surface ex vivo: bone reflection (ONH)				
P_+ [MPa]	t_r [μs]	t_+ [μs]	P_- [MPa]	t_- [μs]
4.37 ± 0.31	2.71 ± 0.10	2.34 ± 0.09	-1.82 ± 0.30	0.93 ± 0.05
Dolorclast measurements at bone surface ex vivo: no bone (ONH)				
P_+ [MPa]	t_r [μs]	t_+ [μs]	P_- [MPa]	t_- [μs]
4.37 ± 0.07	2.37 ± 0.06	2.65 ± 0.04	-2.29 ± 0.10	1.04 ± 0.03

Table 1. Dolorclast measurements *ex vivo* using ONH. Mean ± SEM.

Two experiments were taken in the hindlimb of a freshly sacrificed rabbit (New Zealand White; 4kg). The ONH tip dimensions are 1.1mm x 40mm. To position the ONH, a large bore needle was used to create a guide hole. For the intramuscular measurement, the hydrophone was inserted from the lateral side and positioned 5mm from the medial skin surface (Fig. 1B). The SW applicator was positioned on the shaved skin surface, parallel to the hydrophone and coupled to the shaved skin using ultrasonic gel to minimize wave transmission loss. The hydrophone was then removed and a second guide hole, leading to the bone corner, was created for the hydrophone. The applicator was aligned to strike the bone slightly posterior, so that some of the wave would be propagated into the muscle and towards the hydrophone (Fig. 1B).

Fig. 3 includes the individual and average waveforms and the key parameters are tabulated in Table 1. Although the primary waveforms and data share similar characteristics for the needle hydrophone measurements with and without the bone in place, when the individual waveforms are considered, there is a characteristic second peak for all of the reflected waveforms, which is not seen on any of the control waveforms (Fishers test, $p = 0.001$). Further, the secondary waves for the reflected measurements are more damped than when the bone is removed. For the membrane measurements, there is a double peak for the measurements taken with the bone in place. However, the peak pressures are dramatically less than those recorded without the bone in place. The two peaks are slightly greater than 1µs apart, which is consistent with the bone being approximately 1mm from the bone surface (*i.e.*, suggestive that it is a reflected wave. Also of note is that the peak pressures for the membrane hydrophone measurements were higher than those recorded with the needle hydrophone.

For the free field measurements, the waveforms were consistent with previously published data. Measurements did not differ in any measured parameters between the free field and tissue measurements. It is known that soft tissue attenuates higher frequencies more than lower frequencies; thus, an electrohydraulically generated wave, with its higher frequency components, would be more attenuated than pneumatically generated waves. It is possible that unique characteristics of the waveform contribute to the tissue response to ESWs. Here, we begin to interrogate these waveforms in tissue and *ex vivo* and demonstrate a characteristic second peak at the periosteal surface. These secondary peaks are attributed to reflected waves or creeping waves that–combined with the incident wave–treat the tissue cells with a unique waveform.

Specific Aim #2

Task 2. Histological evaluation of the thickness and number and type of cells in the ESW-stimulated periosteum and controls

2.a. Histological processing of periosteum from 12 goats which underwent ESW treatment and from 6 sham-treated goats

2.b. Histomorphometric evaluation of the thickness of the periosteum and number and type of cells making up the periosteum

We initially performed a pilot study on 6 goats and found that there were no notable effects ESWs in stimulating the proliferation of periosteal cells as we had hypothesized.

Unfocused ESWs (Fig. 4) were initially investigated because of their potentially more desirable safety profile when compared to focused ESWs. Focused ESWs expose tissues around the target site to what may be unwanted exposure to ESWs. Table 2 shows the doses of unfocused shock waves employed. The ESWs were applied to the periosteum in the proximal medial aspect of the tibia, and to the mandible and maxilla.

Table 2. Doses of unfocused ESWs employed in the pilot experiment, n=1.

Goat	Dose mJ/mm2 x no. of shocks
1	0.4 X 3000
2	0.4 X 2000
3	0.4 X 1000
4	0.3 X 1000
5	0.18 X 1000
6	0.1 X 1000

All 6 goats were sacrificed 4 days after application of the ESWs. The tissues were fixed in formalin and decalcified and embedded in paraffin. Histological evaluation of the tibial periosteum (Fig. 5) showed no effect of the ESWs in stimulating the proliferation of cambium layer cells as had been seen in rats and rabbits. The inflammation was graded based on the following scale:

0: No inflammation
1: 1 or more small (barely visible through 10x objective) inflammatory cell infiltrates
2: 1 or more medium (visible through 10x objective) inflammatory cell infiltrates
3: 1 or more large (immediately visible in 10x objective) inflammatory cell infiltrates
4: Tissue necrosis and/or granulation tissue formation
5: Abscess

Hemorrhage was graded with the following scores:

0: No hemorrhage
1: 1 or more small (barely visible through 10x objective) hematomas
2: 1-3 or more medium (visible through 10x objective) hematomas
3: >3 or more medium (immediately visible in 10x objective) hematomas
4: 1 or more large (immediately visible in 10x objective) hematomas

The results are shown in Table 3

Table 3. Scores for the degree of inflammation and hemorrhage.

Dose	Inflam. Score	Hemorrhage Score
0.4X 3000	4	4
0.4 X2000	2	4
0.4X 1000	3	4
0.3X 1000	3	3
0.18X1000	2	2
0.1 X 1000	2	4

Task 3. Determination of the chondrogenic potential of ESW-stimulated cambium cells *in vitro*

3.a. Isolate cells from the enzymatically-digested ESW-stimulated periosteum and sham-treated controls, 4 days post-ESW treatment, and grow in monolayer

3.b. Produce pellet cultures of the periosteal cells in chondrogenic medium
3.c. Process pellets for histological evaluation
3.d. Histomorphometric evaluation of the pellet

Because there was no evidence of the effects of ESWs on periosteal cells in vivo, we used goat marrow-derived MSCs.

We implemented cultures of MSCs in suspension and in hydrogels as tissue simulants in an effort to: 1) enable us to compare the chondrogenic and osteogenic potential of cells exposed to ESWs *in vivo* with cells exposed to ESWs *in vitro*; and 2) to screen the effects of the various shock wave parameters *in vitro* prior to the animal evaluations. We used this *in vitro* system to evaluate the effects of various types of shock waves at various doses on the cell viability and proliferation of MSCs, using platelet-derived growth factor (PDGF)-BB as a control stimulation.

Materials and Methods

Experimental Design

A gelatin-hydoxyphenylpropionic acid (Gtn-HPA) hydrogel was prepared as a 3-dimensional matrix to be seeded with the ESW-stimulated and non-stimulated cells to investigate selected cell behavior. The 2% (by wt.) gel was cross-linked with 0.1 U/ml horseradish peroxidase (HRP) and 1.2 mM hydrogen peroxide (H_2O_2). Bone marrow-derived mesenchymal stem cells (MSCs) serving as periosteal cell surrogates were isolated from bone marrow aspirates of adult Spanish goats (10^5cells/ml bMSCs in the experiments). Focused ESWs (Piezoclast EMS) were used at the following doses:

ESW1: 0.1mJ/mm^2 x 500 impulses

ESW2: 0.4mJ/mm^2 x 500 impulses at 8Hz.

ESW treatment was applied to bMSCs in suspension in a test tube, and the cells subsequently seeded into the hydrogel. bMSC viability in the Gtn-HPA gel containing control and treated bMSCs was determined using a fluorescent live/dead assay (Calcein AM/EthD-1) at day 1.

The capability of the cells to proliferate was evaluated using a proliferation assay. The bilayer hydrogel construct consisted of a Gtn-HPA gel bottom layer containing 1000ng/ml PDGF-BB and a collagen gel seeded with 10^5 cells/ml bMSCs on top. MSC proliferation was measured using DNA PicoGreen assay at days 0, 1, 4, 7 and 14.

For a differentiation assay, Gtn-HPA-encapsulated bMSCs were induced into osteogenesis via osteogenic medium for 21 days; cryosections were then stained with Von Kossa and Alizarin Red to examine mineralization.

Bone Marrow-Derived Mesenchymal Stem Cell Isolation

Bone marrow aspirates were collected from the iliac crest of adult Spanish goats. Cells were suspended in "expansion medium" [DMEM-LG containing 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin/streptomycin (Invitrogen), with the supplement of 10 ng/ml recombinant human fibroblast growth factor-2 (FGF-2; R&D Systems)], and then expanded in a monolayer flask at 37C. Second passage cells at 80-90% confluence were collected to be used for different groups of the experiments.

Gelatin (Gtn) Hydroxyphenyl Propionic Acid (HPA) Hydrogel

A gelatin (Gtn) hydroxyphenyl propionic acid (HPA) hydrogel, which we are using in other studies, was employed here a tissue simulant for the 3-D investigation of cell differentiation. Gtn-HPA 2% was cross-linked with 0.1 U/ml horseradish peroxidase (HRP) and 1.2 mM hydrogen peroxide (H_2O_2). Gtn-HPA was prepared by dissolving 2% Gtn-HPA polymers into 50% of DPBS and 50% of cell-loaded expansion medium (Dulbecco's Modified Eagle Medium-low glucose, DMEM-LG; Invitrogen). The polymer cross-linking was initiated with 0.1U/ml horseradish peroxidase (HRP; Wako Chemical, USA) and 1.2mM H_2O_2 (Sigma-Aldrich) [3]. MSCs in expansion medium were added to the Gtn-HPA prior to the gelation process, at a cell density of 1×10^5 cells/ml. In the control groups, cells received no treatment.

Extracorporeal Shock Wave Treatment

MSC's suspension was centrifuged in 15 ml tube then placed into a distilled water chamber as a conducting medium for shock wave application. SW apparatus installation was maintained to centralize the focal distance from the projectile to the cell pellets. Focused shock waves of different energy flux was delivered using focused shock wave apparatus, with energy flux1 (ESW1: 0.1mJ/mm^2 x 500 impulses), and energy flux2 (ESW2: 0.4mJ/mm^2 x 500) all impulses delivered be at 8 Hz. Cells were re-suspended to be seeded in the gel construct.

Mesenchymal Stem Cell Viability

Viability test was performed 24 hours post-gelation using the Live/Dead assay. Viability/cytotoxicity kit was used (Molecular Probes, Invitrogen) with calcein acetoxymethl easter (Calcein AM) to bind the live cells and ethidium homodimer-1 (EthD-1) to bind the dead cells at the final concentration of 2 µM and 4µM, respectively. After adding the reagents, gels were incubated for 45 min at 37℃. Afterward, gels were washed by adding 3 ml of DPBS to replace the reagents for 30 min. The cells within the gel construct were imaged by fluorescent microscope (Olympus BX60, Japan) for live and dead cells count.

Differentiation Assay

Cells in monolayer and seeded into Gtn-HPA were grown in osteogenic medium for 21 days; gel construct were fixed using 4% PFA for 2 days. Cryosections of each gel construct underwent different staining procedures. Staining of Von Kossa and Alizarin Red was performed to microscopically examine mineralization within the gels.

Effects of Shock Wave-Conditioned Medium MSC Proliferation

Second passage MSCs harvested from same animal were collected at 80-90% confluence; cells were divided into designated groups then seeded into 6-well plates with cell density of 2000 cells/ cm^2.

Group 1	MSC controls; non-shocked cells in normal expansion medium
Group 2	MSCs expanded under a conditioned medium extracted from shock wave-treated cells
Group 3	SW-treated cells kept in their medium

At Day 1, as control group, Group 1 received 3 ml of expansion medium DMEM-LG containing 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin/streptomycin (Invitrogen),. Group 2 received 3 ml of shock wave conditioned medium that was extracted from shock wave treated cells (500 impulses, EFD 0.4 mj/mm^2). Group 3 MSCs treated with SW (500 impulses, EFD 0.4 mJ/mm^2) as described in section. All groups were incubated at 37C with 5% CO_2. Medium was half changed every other day.

At day 3, light microscopic images were taken for cells count. At day 6, cells were fixed by 4% paraformaldehyde at 4C overnight, followed by nuclei staining with 4'6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen Molecular Probes, USA). Fluorescent microscopic (Olympus BX60, Japan) images were taken for cell counting.

Effect of Shock Wave Treatment on the Osteogenic Potential of MSCs

To examine the effect of SW on bMSC stemness, similar methodology as the above experiment (Groups 1 and 3) with cell density of 4000 cells/cm^2. A surface antigen, CD105, known to be expressed by stem cells was evaluated by immunohistochemistry.

Group 1 (control) was MSCs and Group 2 was shock wave-treated MSCs. At days 0 and 7, both groups underwent a fluorescence staining using CD105 (1/100 dilution, ab156756, Abcam, USA) and counter-staining using nuclei staining with 4'6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen Molecular Probes, USA). Fluorescent microscopic imaging was performed at day 0 and 7 with a 10x-objective.

Osteogenic Differentiation of bMSC in Monolayer; Identification and Quantification

To identify and quantify the osteogenic differentiation of 3 groups of MSCs in monolayer, MSCs were seeded in chamber glass slides (Nunc™ Lab-Tek™ chamber slide system, Thermo Scientific, USA) with the density of 2000 cells/cm^2 per chamber. Group1 contained MSCs with normal expansion medium; Group 2 was shock wave-treated MSCs with its SW medium that was replaced by osteogenic medium after 24 hours; and Group 3 was MSCs with normal expansion medium that was replaced by osteogenic medium after 24 hours as well. Expansion medium was replaced every other day for Group 1, for Groups 2 and 3, osteogenic medium was replaced every 3 days. At day 14, cells were rinsed with PBS then fixed with 4% PFA at 4C for 30 minutes. Rinses with PBS followed by ddH2O were performed for all cultures followed by 40mM alizarin red staining (Wako Pure Chemical Industries, Ltd.) for 3 minutes at room temperature; alizarin red staining reveals divalent cations, including calcium. Cultures were then thoroughly rinsed with ddH2O and PBS to remove non-specific staining. Microscopic imaging was performed then the quantification process carried out. CPC (cetylpyridinium chloride) (Wako Pure Chemical Industries, Ltd) dissolved in 10 mM sodium phosphate buffer (pH 7.0) was added to the cultures for 1 hour at room temperature. AR-S extracts were diluted 10 times in 10% CPC solution. The AR-S content of each sample was quantified using plate-reader at absorbance of 562nm. Cell preparations were also stained for calcium using von Kossa stain.

Results

Cell Viability after ESW stimulation

MSCs encapsulated within the Gtn-HPA gel survived the HRP and H_2O_2 induced cross-linking process (blank group in Fig. 6). bMSCs stimulated by ESWs and PDGF-BB also remained viable in the gel (Fig. 6). While there was a decrease (of about 10%) in the number of viable ESW-stimulated cells after the first 24 hours, the large variability questions the meaningfulness of the reduction in viability.

MSC Proliferation

Of note, after 7 days, MSCs stimulated by ESW2 alone proliferated slightly more than cells stimulated by PDGF-BB (Fig. 7). By 14 days the ESW2-stimulated cells exceeded by 2-fold the number of cells stimulated by PDGF-BB. There were no additive or synergistic effects of the combination of ESWs and PDGF-BB on cell proliferation.

MSC Osteogenic Differentiation in the Gel

Gtn-HPA hydrogels supported the differentiation of MSCs into osteogenic-like cells. Gtn-HPA cross-linking supported the intercellular communication and mineral formation within different layers of the gel construct.

Stem Cell Markers

The MSCs displayed positive staining for the surface antigen, CD105, routinely used as a stem cell marker (Fig. 8 A-C). This positive staining was found on day 1 as control after the MSCs were treated with SW2 (500 impulse of 0.4 mJ/mm^2). At day 7, both control and SW2 treated cells stained negative for CD105, which indicates their differentiation into select cells.

Effect of SW2–Conditioned Medium on MSCs Proliferation in Monolayer

Exposure of MSCs in monolayer to SW-conditioned medium increased the proliferation of MSCs compared to the control. All groups substantially increased in cell numbers by more than 3- fold from 3-6 days in culture (Fig. 9). Two-factor ANOVA demonstrated statistically significant effect of groups ($p=0.036$; power= 0.63) and time in culture ($p < 0.0001$; power=1) on the number of MSCs. Fisher's PLSD *post hoc* test showed that there was a statistically significant difference between the number of MSCs treated with SW-conditioned medium (Group 2) and the MSCs in the control expansion medium (Group 1; $p=0.01$). There was no statistically significant difference between Group 2 and 3.

After 3 days there was a statistical significant effect of SW-conditioned medium on the number of cells in culture (an increase of 25%; one factor ANOVA, $p=0.024$; power= 0.64)

After 6 days, there was around 20 % increase in proliferation of the MSCs in SW-conditioned medium but was not statistically significant by one-factor ANOVA, (p=0.06, power 0.44)

At 3 days, there was no statistically significant effect of SW treatment when comparing the proliferation of non- shocked MSCs grown in SW-conditioned medium and shocked cells grown in their own medium (p=0.3%; power 0.13). There was no statistical difference between these 2 groups at 6 days (one-factor ANOVA, p=0.1 %, power=0.25).

MSC Osteogenic Differentiation

Examining the calcium formation reflecting osteogenic differentiation in monolayer, SW2-treatd cells grown with osteogenic medium showed significantly higher calcium formation compared to MSCs grown in expansion medium (Fig. 10). Osteogenic medium-SW2 cells showed a greater amount of calcium formation than non-shocked cells in osteogenic medium (Fig. 10), but this finding was not statistically significant.

Gtn-HPA 2% hydrogels supported the differentiation of MSCs into osteogenic-like cells (Fig 11 A-F). Gtn-HPA cross-linking supported the intercellular communication for mineral formation within different layers of the gel construct.

Discussion

This set of experiments demonstrated that Gtn-HPA hydrogels could provide a supportive environment for MSC survival, proliferation, and differentiation of cells stimulated by ESWs. A notable finding was that after 14 days, ESWs alone stimulated a 2-fold greater proliferation of MSCs compared to PDGF-BB. ESW treatment of bone marrow (and periosteum) could increase greatly the number of osteogenic cells for boner reconstruction. Gtn-HPA hydrogels are commended by their tunable cross-linking and preservation of the bioactivity of growth factors such as PDGF-BB.

These experiments also demonstrated that MSC treated with ESWs release a paracrine factor that stimulates the proliferation of MSCs. ESW treatment did not effect the osteogenic differentiation of MSCs.

Specific Aim #3

Task 4. Evaluation of the cartilage repair induced by ESW-stimulated periosteum in a goat model

4.a. ESW stimulation of the periosteum in the proximal tibia
4.b. Harvest of the ESW-treated periosteum and sham controls after 4 days, and implantation into chondral defects in the trochlear groove of the same animals
4.c. Sacrifice of the goats 16 weeks post-implantation, and processing of tissue for histological evaluation.
4.d. Histomorphometric evaluation of the cartilage repair

This aim is to be achieved in future experiments.

Because there were no notable effects seen in the ESW stimulation of periosteum compared to the non-ESW-stimulated control, this task was not completed as originally proposed. Instead the effects of ESW on marrow was investigated.

Implantation of Shock Wave-Stimulated Periosteum in a Cartilage Defect in the Goat Model

A pilot study (Table 4) was performed to determine the effect so the ESWs on the periosteum of the proximal medial tibia of goats (goat #1), and to implant shock wave- stimulated periosteum imto a standardized shallow osteochondral defect in the goat knee. Spanish goats (ages 1.5 to 3 years) weighing approximately 25 kg were used in this study. Prior to surgery, the stifle (knee) joints was roentgenographically examined to exclude animals with degenerative joint disease or other noted orthopedic problems.

All operations were performed under general anesthesia and sterile conditions. The knee joints was opened by an anteromedial approach and the patella was luxated laterally to expose the trochlea. One shallow osteochondral defect, ~10 mm x 10 mm, was produced in the middle of the trochlear groove; the defect thus covered all the articulating surface of the patellofemoral articulation. The outlines of the defect were first be marked (Fig. 12A) and then the articular cartilage was scraped from the bone using a curette and loupe visualization. Osteotomes were used to remove the calcified cartilage layer and subchondral bone down to a depth of ~1 mm below the tidemark (Fig. 12A). The periosteum harvested from the site exposed to the shock

waves was sutured into the defect (Fig. 12B). The knee joints were immobilized for 8 days using an external fixation apparatus. One goat (goat #2) was sacrificed at 8 days and one goat (#3; Fig. 12C) was sacrificed 14 days after implantation (Fig. 12D). The distal femurs were resected and processed for histological evaluation, which is in progress.

Table 4 Pilot goats procedures. Shock waves applied to the proximal medial tibia. The dose was 3000 shocks at 0.41 mJ/mm^2 at 4Hz.

Goat	Purpose	Type
1	Periosteum donor site at time of implantation (4 days)	
2	Osteochondral defect with ESW-stim periosteum, 8 days f/u (pin removal)	R: Osteochondral defect in intertroclear groove covered with stimulated periosteum
3	Osteochondral defect with ESW-stim periosteum, 2 weeks f/u	R: Osteochondral defect in intertroclear groove covered with stimulated periosteum

The results revealed no notable difference among groups

Effects of Shock Waves on Goat Marrow *In Vivo*

In a pilot study of 2 goats, we applied 1000 (goat 1) and 3000 (goat 2) shocks of 0.3mJ/mm^2 of ESWs to the proximal tibia, and evaluated the histological changes in the marrow after 4 days and 4 weeks (Fig. 13). The marrow at this site was fatty marrow (Fig. 13A, B) facilitating the clear identification of the distinctive changes in the marrow in the focal zone of the ESWs after 4d and 4wk (Fig. 13C, D). There was a dramatic cell proliferative response after only 4 days (Fig. 13C). The non-lethal changes were reflected in part by the intact vascular structures and the granulation tissue and well-differentiated stroma after 4 wks. There were no notable differences between 1000 and 3000 shocks. The cellular make-up of the marrow at this location included adipocytes, and stromal and vascular cells. The cellular changes induced at 4d by the shock waves were restricted to the focal zone and likely reflected the effects of the ESWs on the proliferation of stromal and vascular cells (Fig. 13C), probably indirectly through the induction of the release of anabolic factors including vessel endothelial growth factor (VEGF). ESW-stimulation of these cell types resulted, at 4 wks, in neovascularization and formation of reparative tissue (Fig. 13E-L). We did not expect to see cartilage induced in the marrow environment by the ESW treatment. Our supposition is that these marrow stromal and vascular cells which proliferate and are markedly stimulated under the influence of ESW, after only 4 days, will gain access to the cartilage defect through the microfracture holes and will form cartilage under the influence of local cues. In the proposed project we will perform a thorough evaluation of the cell types stimulated by ESWs.

Of note when examining Figs. 13C and D, which clearly show the dimensions of the ESW focal zone, is that we have applicator heads for our SW apparatus that allow us to control the location (ahead of the head) and length and diameter of the focal zone.

III. KEY RESEARCH ACCOMPLISHMENTS

- ESWs in the range of doses employed in the study do not stimulate the proliferation of periosteal cells in the goat as they do in the rat and rabbit.
- ESWs stimulate a regenerative response in goat marrow *in vivo*.
- *In vitro* ESWs stimulate the proliferation of MSCs.

IV. REPORTABLE OUTCOMES

ESWs show promise as a method for the noninvasive stimulation of marrow for procedures such as microfracture for cartilage repair

V. CONCLUSIONS

ESWs are not likely to be of use for the stimulation of periosteum to be used as an autograft for cartilage and bone reconstruction.

VI. REFERENCES

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2. Kearney CJ, Hsu HP, Spector M. The use of extracorporeal shock wave-stimulated periosteal cells for orthotopic bone generation. *Tissue Eng Part A* 2012; **18**(13-14): 1500-8.
3. Hu M, Kurisawa M, Deng R, et al. Cell immobilization in gelatin-hydroxyphenylpropionic acid hydrogel fibers. *Biomaterials* 2009; **30**(21): 3523-31.

VII. APPENDICES

None

Fig. 1 (A) Free field setup for the Dolorclast devices: The needle hydrophone is seen in the water bath, connected to the adapted Gorillapod that allowed for flexible positioning. (B)In the first experiment, the needle hydrophone is placed intramuscularly and the ESW applied from the medial side, with the wave front perpendicular to the needle. In the second setup, the needle is positioned at the tibial bone corner and the ESW device angled so that the wave strikes both the bone and the needle.

Fig. 2 Experimental setup for measurements of the waveform around bone *ex vivo*. The shock wave source is pointed at the medial tibia bone surface in a water bath and the needle hydophone positioned to capture the reflected wave.

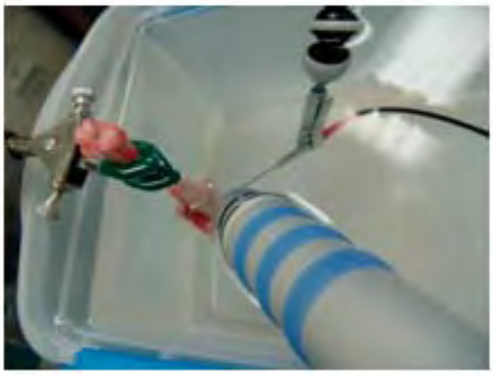
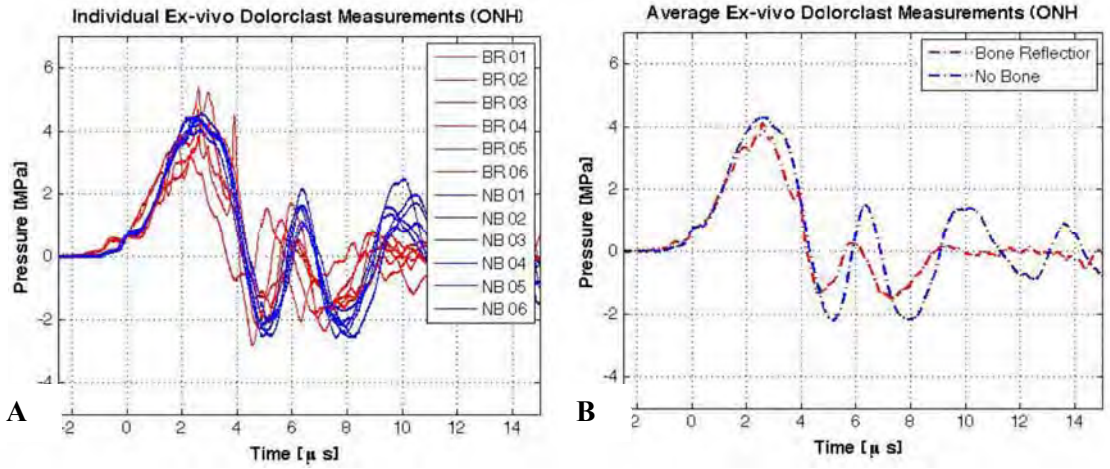


Fig. 3 (A and B) Individual and average measurements for Dolorclast bone reflection using the ONH. The individual waveforms with the bone present demonstrate a characteristic second peak in the compressive wave, which is attributed to secondary reflected or creeping waves from the bone.



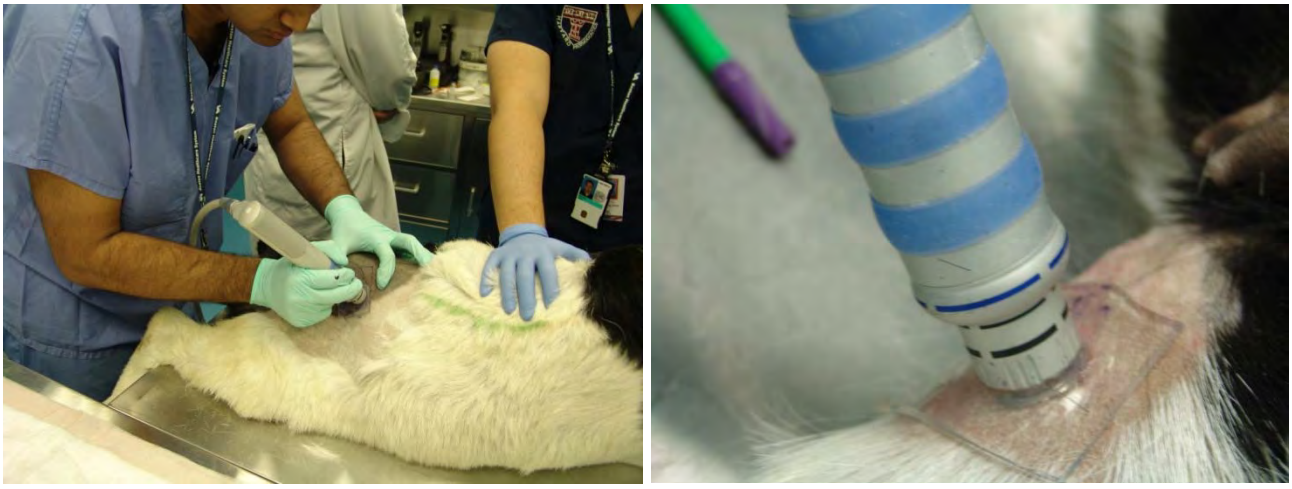


Fig. 4. Unfocused shock waves were applied to the skin overlying the periosteum of the proximal tibia.

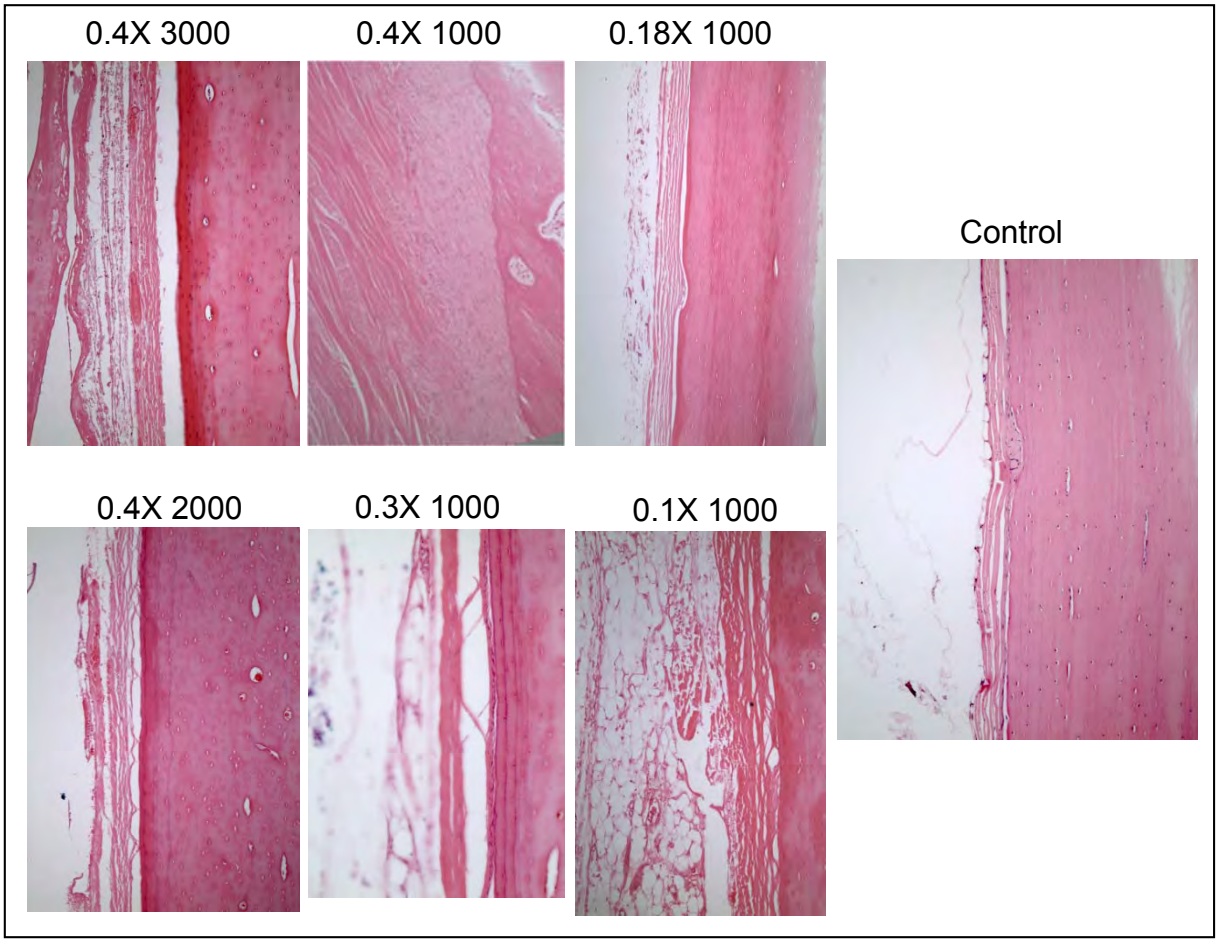


Fig. 5. Histology of the tibial periosteum in the animals treated with different doses of unfocused ESWs.

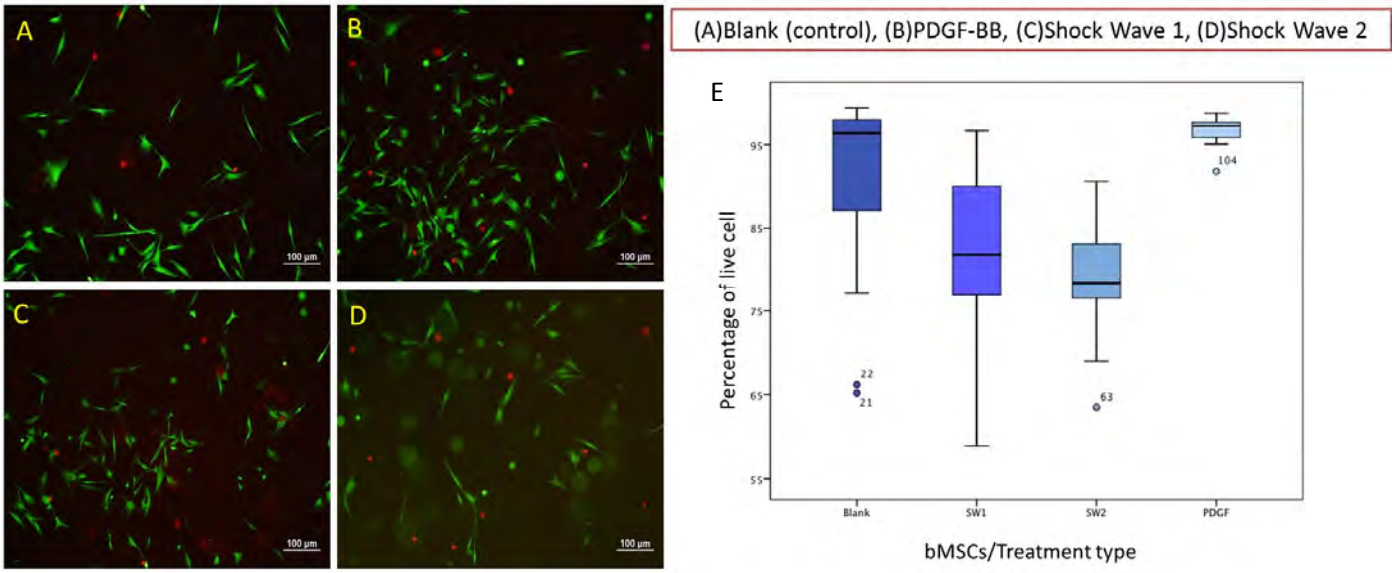


Fig. 6 (A-D), Live/dead fluorescence images of Calcein AM (green) indicating live MSCs and AthD-1 (red) indicating dead MSCs seeded in a GTN-HPA gel, 24 hours post-stimulation with ESWs and PDGF-BB as a control. (E), 24-hour viability assay, bMSCs in 2 % GTN-HPA of different groups (n=4).

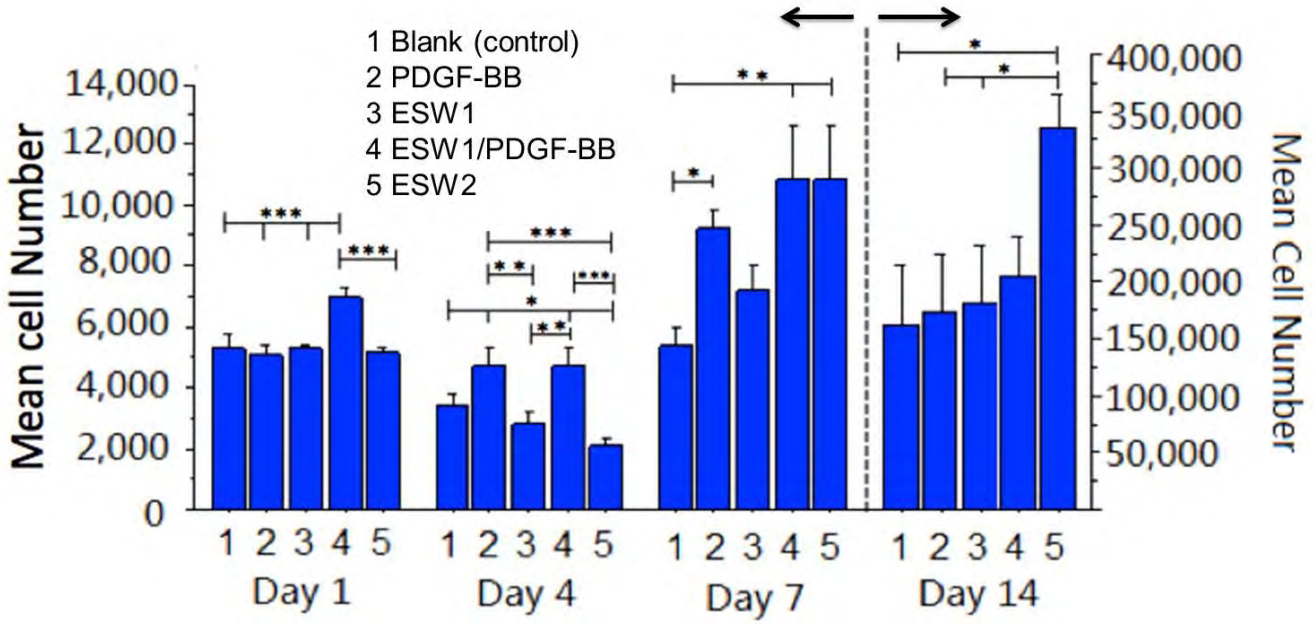


Fig. 7. Effect of different stimulants on bMSC proliferation in Gtn-HPA hydrogel constructs at days 1, 4, 7, and 14. (n=6).

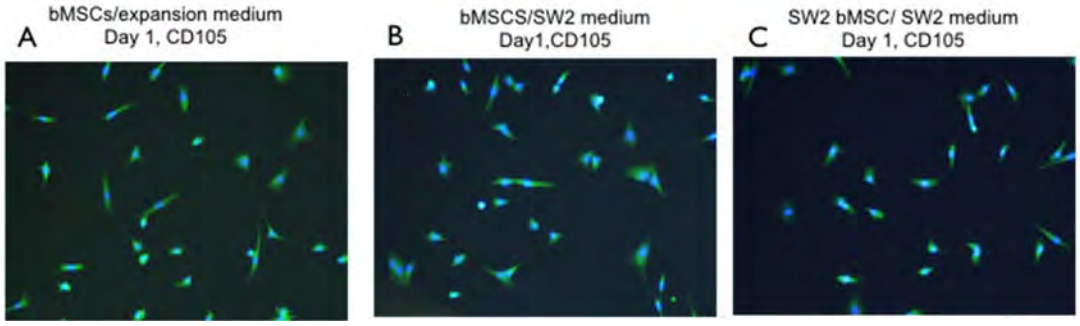


Fig.8. Fluorescence Images for CD105 at day 1, A) bMSC in expansion medium (control) B) bMSCs in SW conditioned medium C) SW treated bMSC in SW conditioned medium (n=4).

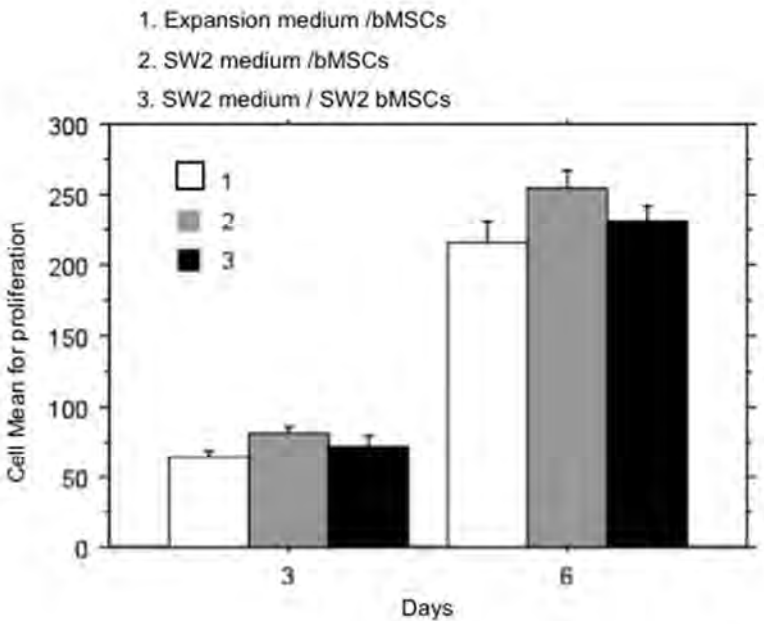


Fig. 9 Effect of SW2 –conditioned medium on bMSC proliferation in monolayer after 3 and 6 days.

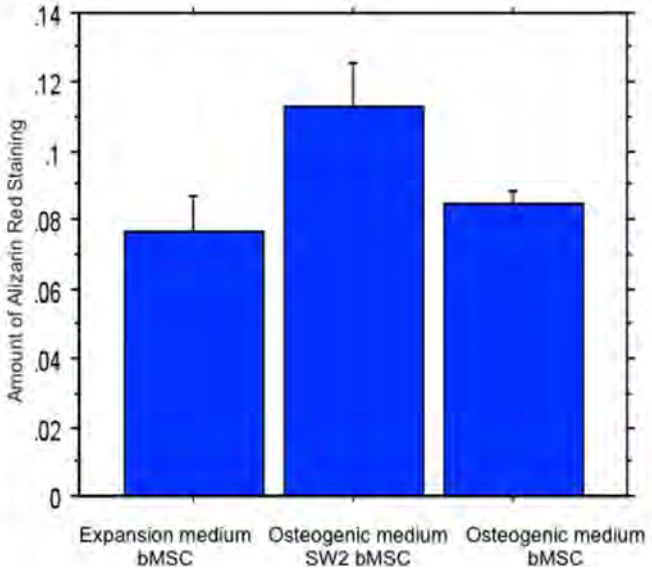


Fig. 10. Effect of shock waves on bMSC osteogenic differentiation in monolayer.

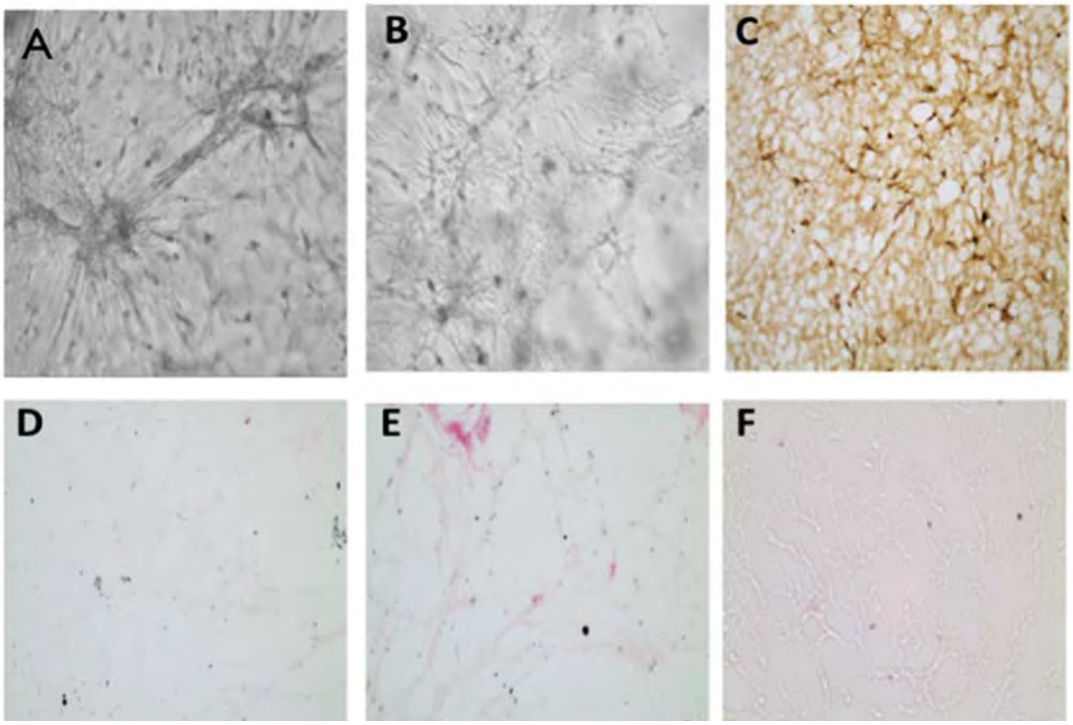


Fig. 11 (A,B) bMSCs encapsulated within Gtn-HPA undergo osteogenic differentiation (C) Alizarin Red staining showing calcium formation within the Gtn-HPA after 21 days. (D-F) Von Kossa staining showing mineralization within the Gtn-HPA gel.

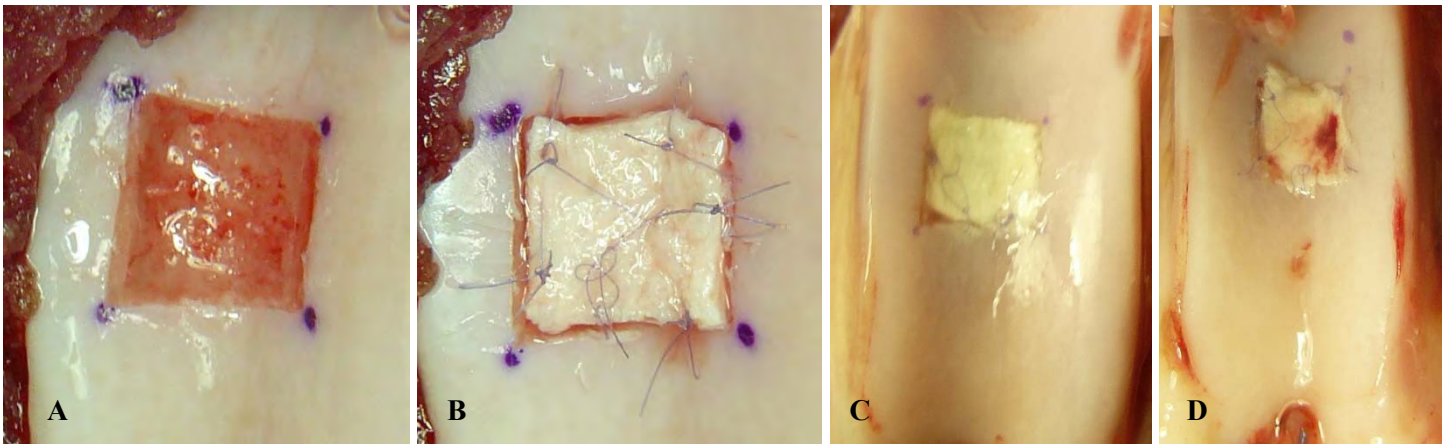


Fig. 12. Photographs of: A) the shallow osteochondral defect prepared in goat #3; B) the shock wave-stimulated periosteum sutured into the defect in goat #3; C) the appearance of the defect in goat #3 at the time of sacrifice; and D) the appearance of the defect in goat #2 at sacrifice.

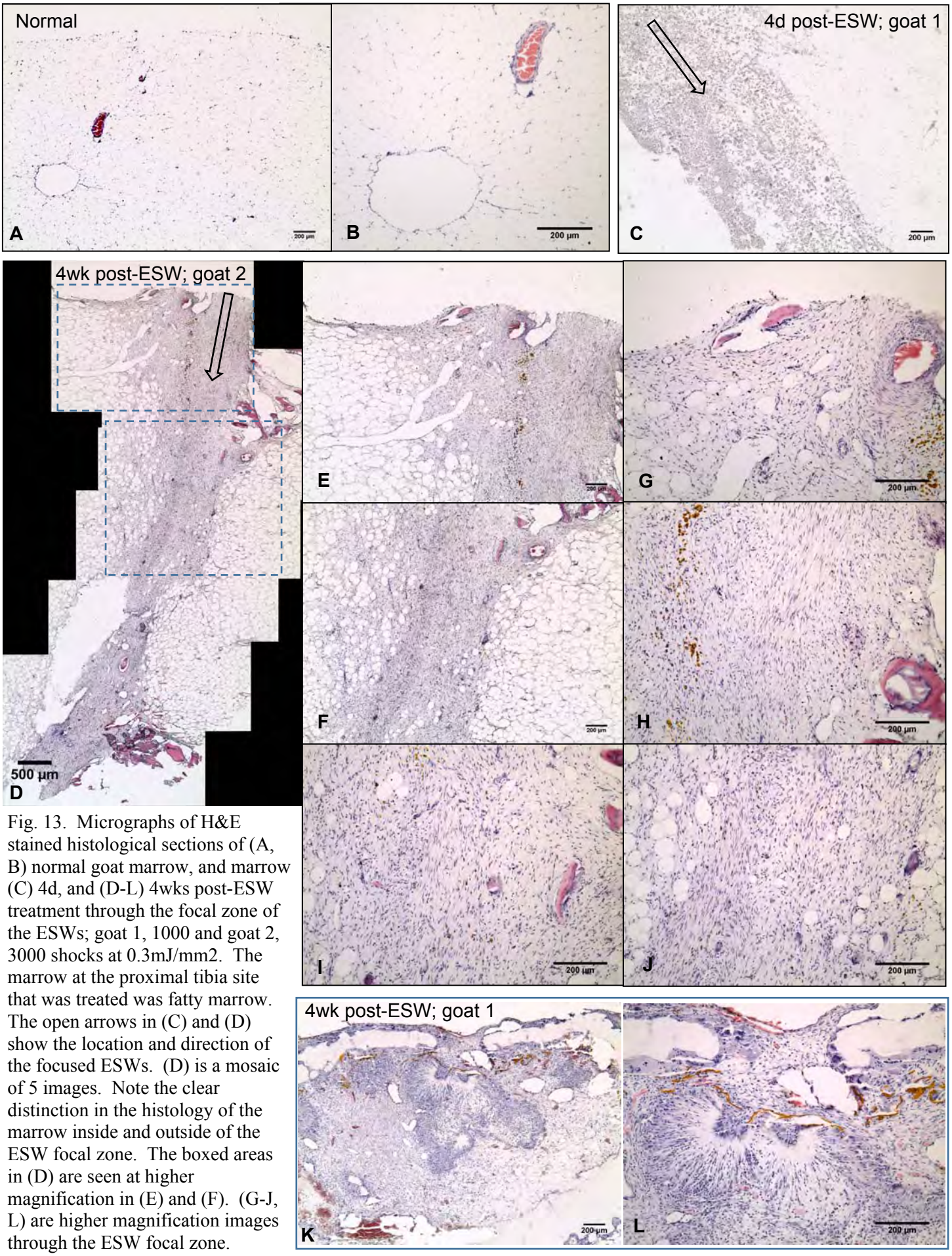


Fig. 13. Micrographs of H&E stained histological sections of (A, B) normal goat marrow, and marrow (C) 4d, and (D-L) 4wks post-ESW treatment through the focal zone of the ESWs; goat 1, 1000 and goat 2, 3000 shocks at 0.3mJ/mm2. The marrow at the proximal tibia site that was treated was fatty marrow. The open arrows in (C) and (D) show the location and direction of the focused ESWs. (D) is a mosaic of 5 images. Note the clear distinction in the histology of the marrow inside and outside of the ESW focal zone. The boxed areas in (D) are seen at higher magnification in (E) and (F). (G-J, L) are higher magnification images through the ESW focal zone.